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Title : Preparation process of blood coagulation factor VII and/or activated blood coagulation factor VII

[Abstract]

[Object of the Invention]

Providing a separation process of FVII and/or FVIIa from FVIIa-ATIII complex, and a preparation process of FVII and/or FVIIa on the basis of the separation process.

[Construction of the Invention]

Separating FVII and/or FVIIa from FVIIa-ATIII complex, by developing a solution containing FVII and/or FVIIa through an anion exchange resin and thereby allowing the FVIIa-ATIII complex to be adsorbed on the resin, and subsequently eluting FVII and/or FVIIa with a solution containing Ca^{2+}.

[Claims]

[Claim 1] A separation process of FVII and/or FVIIa from activated blood coagulation factor VII-anti-thrombin III complex (sometimes referred to as "FVIIa-ATIII" hereinbelow), comprising developing a solution containing blood coagulation factor VII (sometimes referred to as "FVII" hereinbelow) and/or activated blood coagulation factor VII (sometimes referred to as "FVIIa" hereinbelow) through an anion exchange resin.

[Claim 2] A separation process according to claim 1, comprising developing an FVII and/or FVIIa-containing solution contaminated with the FVIIa-ATIII complex through an anion exchange resin, subsequently allowing the FVIIa-ATIII complex to be adsorbed on the resin, and eluting or just passing FVII and/or FVIIa, thereby isolating and purifying FVII and/or FVIIa.

[Claim 3] A separation process according to claim 2, wherein the buffer for eluting or just passing FVII and/or FVIIa contains a Ca^{2+} source at a concentration less than 40 mM.

[Claim 4] A separation process according to claim 2 or 3, wherein the buffer for eluting or just passing FVII and/or FVIIa is at pH 10.0 or less.

[Claim 5] A preparation process of blood coagulation factor FVII and/or activated blood coagulation factor FVII, the preparation process including a process on the basis of a separation process according to any one of claims 1 to 4.

[Claim 6] A blood coagulation factor FVII composition and/or an activated blood coagulation factor FVII composition, the compositions containing substantially no FVIIa-ATIII complex.

[Claim 7] A blood coagulation factor FVII composition and/or an activated blood coagulation factor FVII composition according to claim 6, the compositions being produced by a preparation process according to claim 5.

[Detailed Description of the Invention]

[0001]

[Industrial Field of the Invention]

The present invention relates to the field of plasma protein. More specifically, the invention relates to a preparation process of blood coagulation factor FVII (sometimes referred to as "FVII" hereinbelow) and/or activated blood coagulation factor VII (sometimes referred to as "FVIIa" hereinbelow). In accordance with the invention, it is provided a preparation process of FVIIa at a reduced content of contaminated protein from a plasma fraction containing FVII and/or FVIIa in a very simple manner, the preparation process comprising subjecting a plasma fraction containing FVII and/or FVIIa to anion exchange chromatography.

[0002]

[Prior Art and Problems that the Invention is to Solve]

It has commonly been known that FVII is a vitamin K-dependent blood coagulation factor and functions as an extraneous blood coagulation initiation factor. Like other vitamin K-dependent coagulation factors, FVII contains the Gla region composed of 10 ?-carboxyglutamic acids (sometimes referred to "Gla" hereinbelow) in the amino acid sequence from the N terminus to the 35-th amino acid residue (Proc. Natl. Acad. Sci. USA, vol.183, p.2412-2416, 1986). It is known that FVII is hydrolyzed in vitro at 152Arg-153Ile by activated blood coagulation factor X (sometimes referred to as "FXa" hereinbelow), activated blood coagulation factor IX (sometimes referred to as "FIXa" hereinbelow) or thrombin (sometimes referred to as "FIIa" hereinbelow), so that FVII is converted into activated FVII, namely FVIIa, which is composed of chains H and L crosslinked with one S-S bond (J. Biol. Chem., vol.251, p.4797-4802, 1976).

[0003]

For a supplementary therapy of patients with hemophiliacs A and B, formulations of blood coagulation factor VIII (sometimes referred to as "FVIII" hereinbelow) and blood coagulation factor IX (sometimes referred to as "FIX" hereinbelow) have been administered. However, it has been a significant problem that the therapy has caused the emergence of neutralizing antibody (sometimes referred to as "inhibitor") against FVIII and FIX. A report has been issued, telling that FVIIa is effective for the treatment of patients with the emergence of the inhibitor. Currently, plasma-derived FVIIa and FVIIa of genetic recombination type to be prepared on the basis of the application of the genetic recombination technology are under the progress of development (Japanese Journal of Fundamentals and Clinical Practice, vol.30, p.315-325, 1996; Haemostasis, vol.19, p.335-343, 1989).

[0004]

A process of isolating and purifying FVII from plasma generally comprises thawing frozen plasma under cooling, discarding the precipitate fraction therefrom to recover the supernatant, developing the supernatant through an anion exchange resin and separating a protein fraction with the Gla region (PPSB fraction), and further subjecting the protein fraction to immunoaffinity chromatography. Even by such purification process comprising the immunoadsorbent process with such an extremely high specificity, it is very difficult to purify FVII. The reason why the FVII purification is difficult resides in that FVIIa-ATIII complex is inevitably contained as an impure contaminant protein in the final FVII and in that the FVII purity in an eluted solution by immunoaffinity chromatography never exceeds 85 %. So as to block the activation of blood coagulation factors, the addition of heparin at a purification process is effective. However, it has been reported that heparin then promotes the generation of FVIIa-ATIII complex (J. Biol. Chem., vol.268, p.767-770, 1993).

[0005]

The following two processes are candidates for a method for separating FVIIa-ATIII complex from FVII and/or FVIIa;

[1] immunoaffinity chromatography by using an anti-FVII monoclonal antibody never recognizing FVIIa-ATIII; and

[2] separation by gel filtration.

Nevertheless, the process [1] requires enormous laborious works for the recovery of such monoclonal antibody desirable. The process [2] possibly involves significant reduction of the inter-process yield if the separation is promoted at a far higher level, because the molecular weight of FVIIa-ATIII complex has a distribution within a range of 50 kda to 80 kda. Not any more report has been published yet, about the separation process of FVIIa-ATIII complex from FVII and/or FVIIa, except those processes described above.

[0006]

[Means for Solving the Problems]

The inventors have made research works and investigations in various fields so as to attain the removal of FVIIa-ATIII complex from an FVII fraction. Thus, the invention has been achieved. In accordance with the invention, a purification process of FVII and/or FVIIa is provided, comprising adjusting an FVII- and/or FVIIa-containing solution possibly contaminated with FVIIa-ATIII complex to a Ca^{2+} concentration equal to or greater than 40mM and pH 10.0 or less, and developing the solution through an anion exchange resin, followed by elution with a buffer at a Ca^{2+} concentration equal to or greater than 40 mM and pH 10.0 or less. By the separation process in accordance with the invention, highly purified FVII and/or FVIIa can be prepared.

[0007]

The invention will now be described in detail below.

The starting material to be used in the invention is a solution containing FVII and/or FVIIa, as prepared from plasma or a plasma fraction prepared by appropriate chromatography procedures, or Corn's ethanol separation process or a modification thereof. The optimum starting material is prepared by once purifying plasma crudely by anion exchange chromatography to recover a solution

of a protein with the Gla region (PPSB fraction), developing the PPSB fraction on an anti-FVII monoclonal antibody-immobilized affinity gel and recovering an eluted solution. The purification of FVII and/or FVIIa is not specifically limited to processes based on the immunoadsorption principle, but any process with potential to purify FVII and/or FVIIa is applicable.

[0008]

At the separation process of FVIIa-ATIII complex from an FVII fraction, a solution containing FVII and/or FVIIa is developed through an anion exchange resin to allow the FVIIa-ATIII complex to be adsorbed thereon and then remove the complex. At the process, various conditions can be used. The contact to an anion exchange resin may be carried out by batch process or continuous column process. FVII and/or FVIIa may be either adsorbed or non-adsorbed, provided that the FVIIa-ATIII complex can be adsorbed and removed. Preferably, FVIIa-ATIII complex as well as FVII and/or FVIIa may be adsorbed and removed. The anion exchange resin to be used is not specifically limited. As the resin, preferably, DEAE Sepharose Fast Flow (manufactured by Pharmacia) and Q Sepharose Fast Flow (manufactured by Pharmacia) are used. Any buffer at a Ca^{2+} concentration below 40 mM and pH 10.0 or less can be used as the buffer for the separation of FVIIa-ATIII complex from FVII and/or FVIIa.

[0009]

The solution containing FVII and/or FVIIa, from which the FVIIa-ATIII complex has been removed at the process, is subjected to additional processes for formulation. When FVII is required to be more thoroughly activated into FVIIa, an additional activation process on an anion exchange resin is recommended.

[0010]

The resulting FVIIa may be formulated into a pharmaceutical formulation for therapeutic treatment, diagnosis or other uses. For a formulation for intravenous injection, the composition (the resulting FVIIa composition) is generally dissolved in an aqueous solution containing physiologically

compatible substances, for example sodium chloride and glycine, at a buffered pH compatible with physiologically conditions. From the procurement of pro-longed stability, it is suggested that the final dosage form may be a freeze-dried formulation. The guideline for intravenous compositions has been established and included in the authority's guidelines, for example as "Biological Formulation Standard".

[0011]

Thus, in accordance with the invention, it is provided an industrial-scale separation and preparation process of FVII- and/or FVIIa compositions from plasma or plasma fractions containing FVII and/or FVIIa in a simple manner, along with an FVIIa formulation per se with no substantial contaminant such as FVIIa-ATIII complex, on the basis of the process. The invention will specifically be described below in examples, but the invention is not limited to the examples.

[0012]

[Examples]

Prior to the description of examples, the assay methods of the FVII activity, the FVIIa content and the content of FVIIa-ATIII complex in accordance with the invention are briefly described.

1) Biological activity of FVII

FVII binds to the tissue factor as a blood coagulation initiation factor and then initiates blood coagulation. The assay method of FVII comprises adding a sample to an FVII-deficient plasma and incubating the sample therein for a given period, adding a PT reagent containing the tissue factor, phospholipid and Ca^{2+} to the incubation mixture, counting the coagulation time then and calculating the biological activity on the basis of the coagulation time.

2) Assay of FVIIa content

FVIIa content is assayed by using SDS-PAGE. When activated, FVII (molecular weight of 50 kda) is divided into two chains bonded with one S-S bond. The molecular weight (abbreviated as "MW" sometimes below) of the H chain is 30 kda, while the molecular weight of the L chain is 20

kda. On reductive SDS-PAGE, non-activated substances are detected at the position of MW 50 kda; the H chain is detected at the position of MW 30 kda; and the L chain is detected at the position of MW 20 kda. The detected bands are read with a densitometer: the band at MW 50 kda was defined as indicative of non-activated FVII content in %; and the sum of the contents of the chains H and L was defined as FVIIa content in %. The activation ratio was expressed as a value in percentage (%)of the FVIIa content divided by the sum of the FVII content and the FVIIa content.

3) Assay of the content of FVIIa-ATIII complex

The content of FVIIa-ATIII complex was assayed by using non-reductive SDS-PAGE. The FVIIa-ATIII complex is distributed within a range of 50 kda to 80 kda. The content of molecules of MW 50 kda or larger as detected on the non-reductive SDS-PAGE, namely high-molecular fractions than those of FVII and FVIIa, was read with a densitometer, which was defined as the content of FVIIa-ATIII complex.

[0013]

Example 1

Freshly frozen plasma (100 liters) was thawed under cooling and centrifuged to discard the precipitate fraction. The resulting supernatant was added to an anion exchanger (DEAE-Sephadex A-40, manufactured by Pharmacia), followed by sufficient washing with 20 mM citric acid/0.1M NaCl buffer, pH 7.0 and elution of a PPSB fraction including the Gla region with 20 mM citric acid/0.5M NaCl buffer, pH 7.0. Ten liters of the eluted solution were developed on an anti-FVII monoclonal antibody-immobilized affinity gel preliminarily equilibrated with 50 mM Tris/150 mM NaCl/5.0 mM $CaCl_2$ buffer, pH 8.0, followed by washing with 50 mM Tris/2.5 M NaCl/5.0 mM $CaCl_2$ buffer, pH 8.0 and additional washing with 50 mM Tris/50 mM NaCl/5.0 mM $CaCl_2$ buffer, pH 8.0, and subsequent elution with 50 mM Tris/30 mM NaCl/10 mM EDTA· 2Na buffer, pH 7.4 to recover an FVII fraction. The FVII fraction was developed through a virus removal membrane (Bemberg Microporous Membrane, manufactured by Asahi Chemical Industries, Co.) preliminarily impregnated with 40 mM

Tris/30 mM NaCl buffer, pH 8.0 to recover a filtrate. The purity of the FVII in the resulting filtrate was 85 %.

[0014]

40 ml of the 0.2 mg/ml FVII solution was developed at a linear velocity of 300 cm/hr on a column of a 5.0-mm inner diameter and a 5.0-cm height, packed with QSepharose Fast Flow (manufactured by Pharmacia) preliminarily equilibrated with 50 mM Tris/30 mM NaCl buffer, pH 8.0, followed by washing at the same velocity with a 10-fold-column volume of 1.0 mM Tris buffer, pH 10.0 and subsequent elution with 1.0 mM Tris buffers, pH 10.0 with addition of various concentrations of $CaCl_2$. The eluted fractions were subjected to SDS-PAGE analysis, to assay the content of FVIIa-ATIII complex. The results are shown in Table 1. When "Q" as a strong ion exchanger was used, the removal of the FVIIa-ATIII complex was attained only at a Ca^{2+} concentration of 40 mM or less, under a condition of pH 10.0.

[0015]

[Table 1]

QFF-eluted Ca^{2+} concentration (mM)	FVIIa-ATIII complex content (%)
20	ND
40	ND
50	8.5
60	10

* ND ; below detectable limit.

[0016]

Example 2

35 ml of a 0.2 mg/ml FVII solution prepared as a filtrate through a virus removal membrane in the same manner as in Example 1 was developed at a linear velocity of 300 cm/hr on a column of a 5.0-mm inner diameter and a 5.0-cm height, packed with Q-Sepharose Fast Flow (manufactured by Pharmacia) preliminarily equilibrated with 10 mM Tris/100 mM NaCl buffer, pH 8.5, followed by washing at the same velocity with a 10-fold-column volume of the buffer, and subsequent elution at a

linear velocity of 180 cm/hr with 10 mM Tris/100 mM NaCl/4.0 mM $CaCl_2$ buffer, pH 8.5. The eluted solution of a mixture of FVII and FVIIa at a concentration of 1.0 mg/ml was aged in the liquid phase for 25 hours. The outcome after aging is shown in Table 2. The outcome indicates that the FVIIa-ATIII complex can be removed successfully under conditions such that the eluting buffer is at a Ca^{2+} concentration of 40 mM or less and pH 10.0 or less, when the strong ion exchanger "Q" is used and that FVII is nearly completely activated by the activation on the anion exchange resin and the continuous activation in the liquid phase. Additionally, the present example reveals that the industrial preparation of FVIIa can be attained by using the strong ion exchanger "Q" for the separation of FVIIa-ATIII complex.

[0017]

Table 2

	FVII yield (%)	FVIIa yield (%)	Activation ratio (%)	FVIIa-ATIII complex content (%)	FVII titer specific activity (U/μg)
After aging	2.5	87.0	97.2	ND	40.2

* ND ; below detectable limit.

[0018]

Example 3

40 ml of a 0.2 mg/ml FVII solution prepared as a filtrate through a virus removal membrane in the same manner as in Example 1 was developed at a linear velocity of 300 cm/hr on a column of a 5.0-mm inner diameter and a 5.0-cm height, packed with DEAE-Sepharose Fast Flow (manufactured by Pharmacia) preliminarily equilibrated with 50 mM Tris/30 mM NaCl buffer, pH 8.0, followed by washing at the same velocity with a 10-fold-column volume of 1.0 mM Tris, pH 10.0, and subsequent elution with 1.0 mM Tris, pH 10.0 with addition of various concentrations of $CaCl_2$. The eluted fractions were subjected to analysis on SDS-PAGE, to assay the FVIIa-ATIII complex content. The results are shown in Table 3. When a weak ion exchanger "DEAE" was used, the removal of the FVIIa-ATIII complex could be attained, only at a Ca^{2+} concentration of 15 mM or less, under a

condition of pH 10.0.

[0019]

Table 3

DEAEFF-eluted Ca^{2+} concentration (mM)	FVIIa-ATIII complex content (%)
10	ND
15	ND
25	1.0
50	4.3

* ND ; below detectable limit.

[0020]

Example 4

70 ml of a 0.2 mg/ml FVII solution prepared as a filtrate through a virus removal membrane in the same manner as in Example 1 was developed at a linear velocity of 200 cm/hr on a column of a 5.0-mm inner diameter and a 3.3-cm height, packed with DEAE-Sepharose Fast Flow (manufactured by Pharmacia) preliminarily equilibrated with 50 mM Tris/30 mM NaCl buffer, pH 8.0, followed by washing at the same velocity with a 10-fold-column volume of the buffer, and subsequent elution at a linear velocity of 150 cm/hr with 50 mM Tris/30 mM NaCl/1.75 mM $CaCl_2$ buffer, pH 8.0. The eluted solution of a mixture of FVII and FVIIa at a concentration of 2.0 mg/ml was aged in the liquid phase for 10 hours. The outcome after aging is shown in Table 4. The outcome indicates that the removal of the FVIIa-ATIII complex can be attained under conditions such that the eluting buffer is at a Ca^{2+} concentration of 15 mM or less and pH 10.0 or less, when the weak ion exchanger "DEAE" is used and that FVII is nearly completely activated by the activation on the anion exchange resin and the continuous activation in the liquid phase. Additionally, the present example reveals that the industrial preparation of FVIIa can be attained by using the weak ion exchanger "DEAE" for the separation of FVIIa-ATIII complex.

[0021]

Table 4

	FVII yield (%)	FVIIa yield (%)	Activation ratio (%)	FVIIa-ATIII complex content (%)	FVII titer specific activity (U/μg)
After aging	1.5	95.1	98.4	ND	39.2

* ND ; below detectable limit.